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On protein quality control, myofibrillar myopathies, and neurodegeneration

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CHAPTER 6

Discussion

Melanie Meister-Broekema

In the crowded environment of the cell, protein-protein interactions must be continuously facilitated. Protein misfolding is a frequent and natural occurrence following the emergence of native proteins from ribosomes. Acute stress conditions, such as heat shock and mechanical stress, cause protein unfolding and activate the heat shock response (HSR), leading to the subsequent up-regulation of heat shock proteins (HSPs) to increase in the cellular protein quality control (PQC) capacity. As discussed in chapter 1, the cellular PQC system encompasses a variety of proteins, including various chaperone systems that may either direct misfolded or damaged proteins to cellular folding machines (e.g. Hsp70- & Hsp90 machines), to short-term storage, or to cellular degradation systems (ubiquitin proteasome system, autophagy lysosome system).

1. Client fate;

Determined competitively rather than sequentially?

Traditionally, it has been assumed that folding of misfolded or damaged proteins is attempted prior to their redirection to cellular storage or degradation systems.¹ However, in line with recent findings in the field, the experiments presented in thesis continue to indicate that client fate may not only be determined by the sequential handling of client proteins by dedicated members of folding and degradation machines, but at least also by the chaperone they encounter first.² Multiple factors may influence the likelihood of a client protein to encounter specific chaperones; these may be categorized as follows:

- *Client characteristics*

Structural characteristics of the client, including its tertiary structure (presence of beta sheets, alpha helices, loops, stretches, grooves), exposed hydrophobic or protein-protein interaction surfaces, charges on amino acids, and the presence of specific post-translational signalling molecules (e.g. ubiquitination, methylation, acetylation) may affect the affinity –or accessibility– of client proteins for specific chaperones that either support folding or degradation.³

- *Cellular levels of ‘active’ chaperones and supporting complexes*

Expression levels of protein quality control (PQC) components may differ by cell type, cell compartment, and cellular stress levels. Their expression levels are tightly regulated, not only by transcription factors but also by tertiary proteins or protein complexes that may either keep chaperones in an inactive state or are

required for the activation of chaperones.^{4–11} Furthermore, chaperones usually do not function alone but in elaborate complexes.⁹ Therefore, the regulation of the assembly of these functional complexes or of the components thereof may be rate limiting for certain chaperone functions and thus influence client fate.

The findings discussed in this thesis support the notion that –together– factors underlying client characteristics and the cellular availability of active chaperone complexes determine the client’s fate. Interestingly, evidence indicates that the expression levels of some of these factors are tightly regulated and rate-limiting (e.g. NEF expression levels) in steering client handling, whereas others seem to be subject to less strict regulations (e.g. Hsp70-expression levels) and only increase overall cellular capacity. Furthermore, our findings in Chapter 2 indicate that the presence of certain toxic protein species does not effectively activate the PQC system until late in the protein aggregation process, at which stage the components of the PQC seem to be insufficient to resolve protein aggregation and prevent cell death. Furthermore, as summarized in our review in Chapter 3, different sets of chaperones are required to handle different disease-associated proteins. Finally in Chapter 5, we showed how a single point mutation in BAG3 can lead to subtle biochemical changes in its function that have fulminant dominant negative effects on protein homeostasis, eventually leading to childhood onset myofibrillar myopathy.

2. NEF selection for Hsp70 machines

The complex regulation of Hsp70s becomes especially evident for BAGs, which serve as nucleotide exchange factors (NEFs) for Hsp70s. In chapter 1, we elaborated that the type of NEFs engaged in a Hsp70-machine determines whether a client is (re)folded (BAG2, HSPBP1, HspHs), stored (HSPBP1), degraded by the UPS (BAG1, BAG2, BAG6), or degraded by autophagy (BAG3).^{2,12–16} Which NEF is engaged might be regulated by the availability of NEFs and their affinity for Hsp70, which is higher for BAG3 than for BAG1 or other NEFs investigated in an *in vitro* study.¹⁴ Interestingly, BAG3 seems to enable cells to redirect proteasomal clients to the autophagy-lysosome system,² whereas specific NEF engagement might be related to direct substrate binding properties of certain NEF (HSPHs) or via recruitment of specific NEFs via DNAJs in other cases.

3. BAG3 P209L: Model for pathogenesis

Interestingly, our results in Chapter 5 showed that a single amino acid mutation in BAG3 can lead to small alterations in the processing of ubiquitinated Hsp70-clients, which has fulminant consequences for cellular PQC. Our findings indicate that these effects of the BAG3 P209L mutation, which is located in the second IPV-domain, are not related to a decrease in HSPB8-binding affinity. In fact, complete loss of HSPB8 binding partially negated the DN effects whereas abrogation of Hsp70 completely negated the DN effects meaning that Hsp70 binding is required for toxicity of the mutant. Interestingly, neither the affinity of BAG3 to Hsp70 binding nor BAG3-NEF function was impaired by the P209L mutation. Yet, the BAG3 P209L-mutation resulted in defects in Hsp70-client processing (primary defect) that seem to culminate in co-sequestration of other chaperones and secondary dominant negative effects on overall cellular PQC. Below, I will explain this in more detail and propose an integrated model on how Bag3 P209L may lead to disease, a model that also questions some aspects of the canonical views on the Hsp70 cycle.

3.1 BAG3 P209L; Primary defects

Besides its functions as NEF for Hsp70, BAG3 up-regulation has also been associated with an increased autophagic flux and re-routing of ubiquitinated, proteasomal, Hsp70-bound clients to the autophagy lysosome system in a process called BAG-induced proteasome to autophagy switch and sorting (BiPASS).² Interestingly, we found that the BAG3 P209L-mutation did not affect the BAG3-ability to increase the autophagic flux. Yet, autophagic degradation of polyQ aggregates by BAG3 P209L was strongly impaired and its expression led to a dramatic increase in cellular (poly)-ubiquitin levels and accumulation of ubiquitinated proteins into BAG3 P209L and p62-positive punctate. Still, although the autophagic flux was properly induced and autophagosomes were formed, our findings showing that LC3 does not co-localize with the BAG3 P209L, Ub-positive positive punctae raise the question as to whether ubiquitinated Hsp70-bound clients are actually properly processed and/or delivered into autophagosomes. Interestingly, recent findings from the Gestwicki-group indeed indicate that, next to BAG3-Hsp70-binding, (in)direct binding of BAG3 to the client itself is crucial for proper client processing.(Rauch et al., 2016) Improper BAG3-client binding may result in improper processing of Hsp70-clients following their release from

Hsp70, even under conditions in which BAG3-Hsp70-binding and NEF function are unimpaired. Our *in vitro* data for BAG3P209L (see Chapter 5) also show that its function as NEF is unaffected but that luciferase refolding is not supported. These findings are consistent with a P209L-associated client-processing defect. How and which domain of BAG3 is involved in client binding remains to be expounded. Besides direct client binding, also client binding via small HSP interactions could be involved (see also 3.3 and Figure 1).

How impaired client processing by BAG3P209L affects its role in autophagic degradation still remains an enigma. It is furthermore unclear whether autophagic transport to lysosomes at the MTOC or autophagosomal-lysosomal fusion are impaired in addition to cargo delivery, which will be discussed in the following paragraphs.

3.1.1 *Does the BAG3 P209L mutation result in impaired autophagosomal delivery to MTOC for fusion?*

After cellular cargo is delivered into autophagosomes, these subsequently need to be actively transported to the MTOC via ATP-dependent dynein motor proteins that are capable of “walking” along microtubules.^{17,18} It is thought that BAG3 also may facilitate transport of autophagosomes across these microtubules by binding to dynein through its PXXP domain.¹⁷ The PXXP domain is located adjacent to the second IPV domain of BAG3, which is affected by the BAG3 P209L mutation. Although this mutation does not directly affect the dynein-binding domain of BAG3, little is known about its effects on the secondary structure of BAG3 or on its effects on functional BAG3 interactions. Although we did not directly investigate dynein-binding in Chapter 5, we found that the BAG3^{ΔPXXP}-mutant (which did not induce BAG3 P209L-punctae-formation by itself) in combination with the BAG3 P209L-mutation (BAG3^{P209L-ΔPXXP} double mutant) did not resolve punctae-formation. These results indicate that impaired binding of BAG3 to dynein (and thus autophagosomal transport) is not the likely cause underlying BAG3 P209L-punctae formation. Still, it might be important to identify whether binding of BAG3 to dynein is impaired, which can be done by performing specific dynein pull-down assays and by transfecting cells with fluorescently tagged LC3 and tracking the movement of autophagosomes across microtubules. Co-

staining with vimentin could verify that autophagosomes do not reach the aggresomes at the MTOC in cells expressing BAG3 P209L.

3.1.2 *Does the BAG3 P209L mutation result in impaired lysosome delivery?*

Lysosomes accept bio-materials destined for degradation from multiple cargo vesicles, including endosomes, phagosomes, and autophagosomes.^{19,20} Next to autophagosomal transport to the MTOC, efficient autophagosome-lysosome fusion also requires the transport of lysosomes –which are scattered throughout the cytoplasm under resting conditions- to the perinuclear region.^{19,20} As other components of the autophagy-lysosome pathway, the number, size, and positioning of lysosomes is tightly regulated and adjusted to the constantly-changing cellular needs. Although BAG3 has not been directly linked to lysosomal transport, it may be worth investigating whether lysosomal number, size, and/or transport are affected by BAG3 P209L's pleiotropic, dominant negative cellular effects. Tools like fluorescent lysosomal membrane markers or lysotrackers can be used to investigate whether the cellular expression of BAG3 P209L affects lysosomal trafficking in mammalian cells.

3.1.3 *Does the BAG3 P209L mutation affect autophagosomal-lysosomal fusion?*

Little is known about the exact processes underlying autophagolysosome-formation. What is known is that SNARE-complexes and the cortactin-dependent recruitment of an F-actin network are required for the fusion of autophagosomes with lysosomes.²¹⁻²³ Comparable to what is stated above, there is no evidence directly linking BAG3 to autophagosomal-lysosomal fusion. However, experiments comparing the fluorescent signal in mammalian cells co-transfected with BAG3 P209L and mCHERRY-GFP-LC3 with cells expressing BAG3 WT and mCHERRY-GFP-LC3 could clarify whether or not autophagosomal-lysosomal fusion is affected by the dominant negative effects of the BAG3 P209L mutation.

3.2 BAG3 P209L; Secondary defects

The primary defect of BAG3 P209L seems to be related to the processing of Hsp70-bound client proteins. However, the consequences of this primary defect are manifold and lead to a rapid perpetuating cycle of decline in the overall PQC capacity of the cells as summarized below.

3.2.1 *Does the P209L-mutation lead to a dysregulation of cellular BAG3 protein levels?*

In our experiments, we consistently observed that transfection of plasmids encoding BAG3 WT or P209L into mammalian cells leads to higher BAG3 P209L protein levels than BAG3 WT protein levels. This difference, which –based on our *in vitro* data– is unlikely related to primary structural ? defect of the P209L-mutation, but might have self-perpetuating effects that may explain the rapid progression the disturbance of cellular PQC in the presence of BAG3 P209L.

There are at least two possible mechanisms that may lead to this increase in cellular BAG3 P209L levels: Improper degradation of BAG3 P209L due to defects in its co-degradation during autophagy and transcriptional up-regulation of BAG3 via the activation of HSF-1 via the BAG3 P209L-induced proteotoxic stress. Proteotoxic stress tends to lead to a pleiotropy of events, including the activation of the transcription factor HSF-1.^{4,24} BAG3 is the only stress-inducible BAG protein, meaning that the activation of HSF-1 leads to the up-regulation of BAG3.²⁵ Although this hypothesis requires further investigation, some of our experiments showed that the stress-inducible, HSF-1-regulated, Hsp70-family member, HSPA6 was up-regulated in cells expressing BAG3 P209L, suggesting that HSF-1 is activated in cells transfected with BAG3 P209L.

Experiments potentially answering this question include the analysis of BAG3 and HSF-1-regulated chaperones (e.g. HSPA6) levels and punctae-formation following cellular co-expression of BAG3 P209L with dominant negative HSF-1 or BAG3 P209L expression in HSF1^{-/-} cells. As the activation of HSF-1 is likely not a primary but a secondary defect caused by BAG3 P209L, it is to be expected that the co-transfection of BAG3 P209L with dominant negative HSF-1 or into HSF-/- cells will not impair with punctae formation per se, but instead result in a slower and less progressive increase in BAG3 P209L positive punctae.

3.2.2 *Trapping of chaperones and PQC components*

In Chapter 5, we found that many chaperones locate to BAG3 P209L-, p62-, ubiquitin-positive punctae. These do not only include chaperones that interact with BAG3 itself, such as the small heat shock proteins

HSPB8, HSPB1, and HSPB5, but also other non-BAG3 partner proteins, such as DNAJB1 and DNAJB6. In Chapter 5, we hypothesize that the entrapment of chaperones might lead to their loss of function such that cellular PQC and protein homeostasis in general progressively decline.

3.3. What is the role of HSPB8 in BAG3-Hsp70-client processing?

What is yet to be unravelled is why a single point mutations in the second IPV motif of BAG3 has so much more detrimental effects on cellular PQC than comparable mutations outside the IPV domain.²⁶⁻³¹ Also, the role of HSPB8 normal BAG3-functioning and the relevance of the observed decline in HSPB affinity to BAG3 P209L in disease pathogenesis are not completely clear, yet. The question whether the trimodal interaction of HSPB8-BAG3-Hsp70 and clients is required for proper client processing in BIPASS is an especially interesting question. *In vitro* studies have shown that Hsp70 may refold HSPB-client proteins³²⁻³⁵ while previous results from our research group indicate that HSPB8 is required for the BAG3-induced autophagic degradation of poly glutamine aggregates.^{12,36} Furthermore, recent results from the Gestwicki laboratory indicate that BAG3 serves as a stress-inducible modular scaffolding protein that binds Hsp70 via its BAG-domain and HSPBs via its' two IPV domains, thereby generating a powerful refolding machinery.³⁷ Interestingly, these two protein interactions do not seem to be influenced by one another, which also reflects our findings that the absence of the IPV domains does not influence BAG3-Hsp70 interactions and that the absence of the BAG domain does not influence BAG3-HSPB8 interactions (see Chapter 5).² In addition, literature suggests that BAG3 and Hsp70 form a stable complex that only transiently interacts with HSPB8.³⁸ Our findings in Chapter 5 indicate that this ternary interaction might be of importance for BIPASS as well. Since deletion of the IPV domains alone does not result in the accumulation of ubiquitin-positive punctae, these results might suggest that not only the formation of this ternary complex, but also the timing of complex formation might be crucial. Whereas BAG3 might be required to 'activate' HSPB8, it is fathomable that the complex then dissociates for BAG3 to function in BIPASS. HSPB8 might then be required again to strengthen the pulling force of BAG3 to Hsp70 and the client itself³⁸ when the LC3-p62-positive

complex is formed, which would be consistent with previous findings in from our laboratory². Figure 1 visualizes this hypothesis.

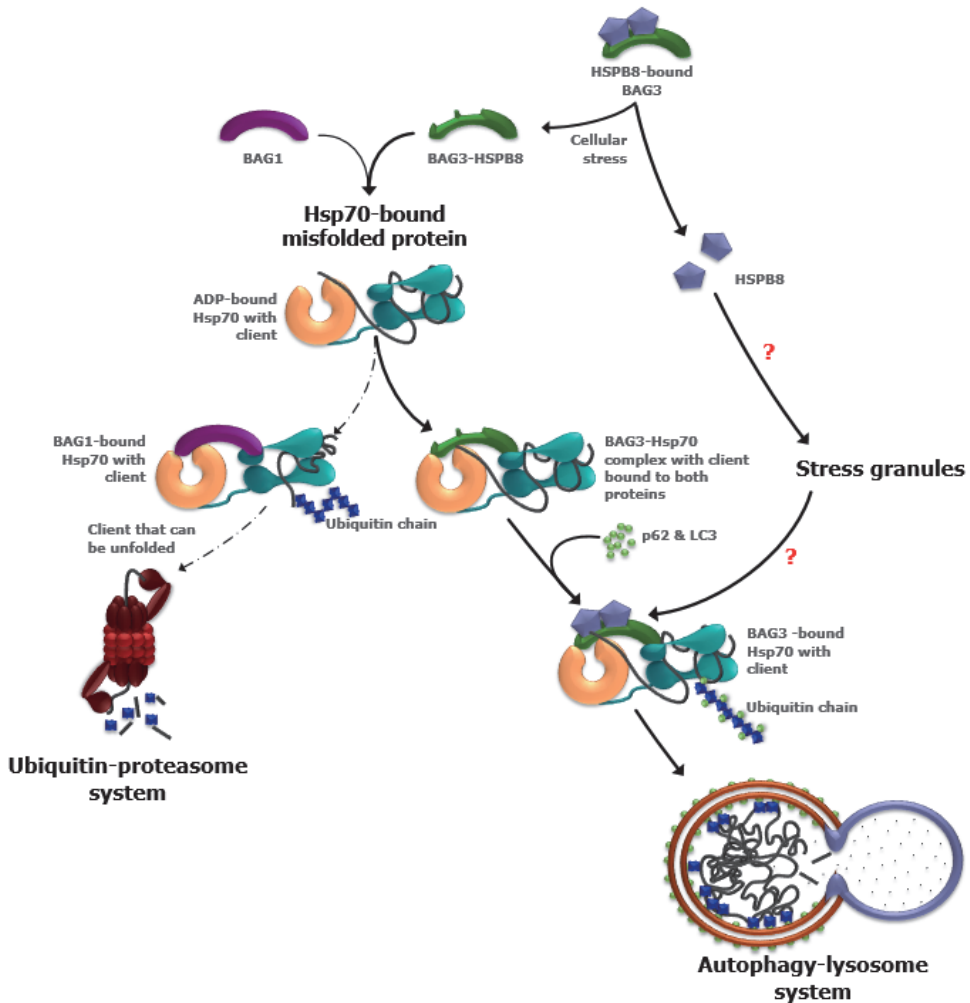


Figure 1: Potential requirement of HSPB8 for BAG3-client-HSP70 binding in BiPASS. HSPB8 might potentially be required to enhance the pulling-force of the BAG3-Hsp70-client complex and thus crucial for the targeting of BiPASS-client proteins to autophagy.

The Gestwicki-group found that the ternary HSPB8-BAG3-Hsp70-complex only promotes refolding when added at specific ratios *in vitro*.³⁹ This might explain how a slight decrease in BAG3-HSPB8-binding affinity –as is the case for the BAG3 P209L mutation– may decrease the pulling force between BAG3 and the

Hsp70-bound client, thereby leading to incomplete or improper client processing. In addition, increased BAG3 levels may further negatively impede on cellular reactions. In line with this hypothesis, we indeed found that excess BAG3 levels lead to re-folding inhibition (Chapter 5). Together, these reactions may further accelerate the self-perpetuating cycle of cell stress, eventually leading to the aberrant activation of BiPASS and accumulation of PQC components into BAG3 P209L-, p62-, ubiquitin-positive punctae and eventual cell death (Figure 2.)

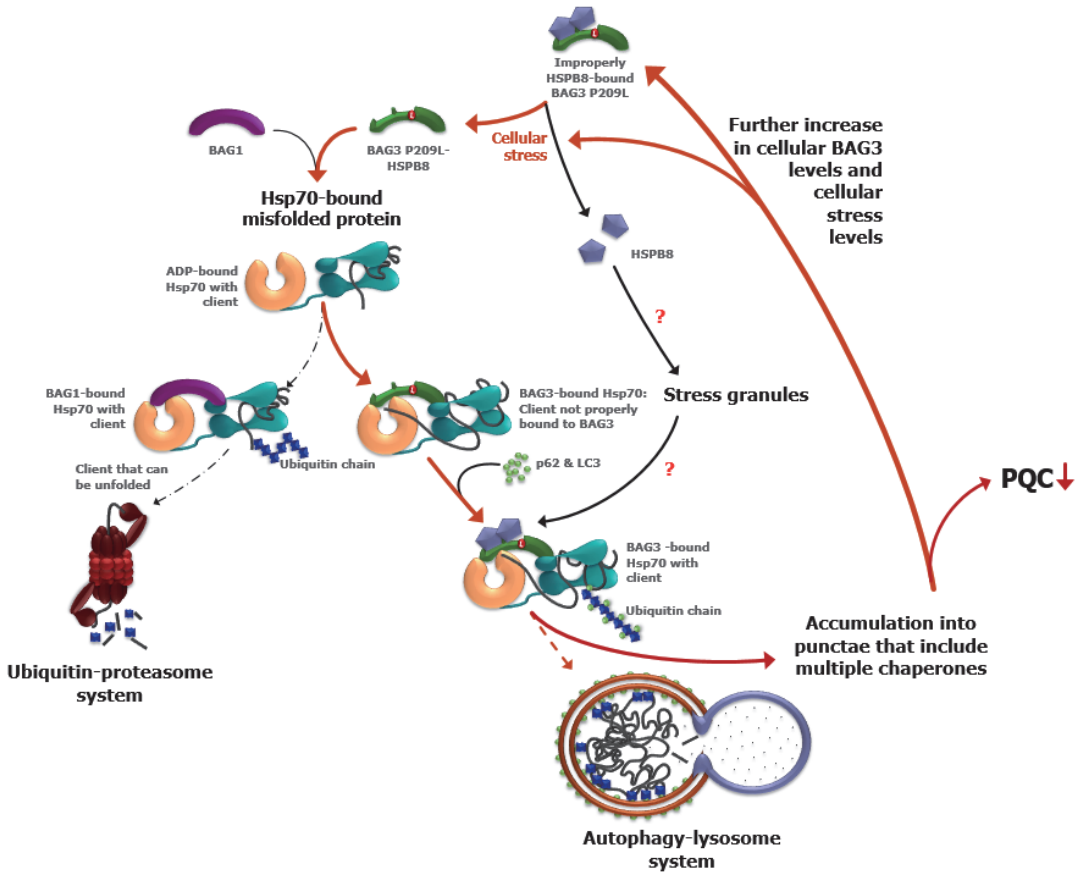


Figure 2: Dominant negative effects of BAG3 P209L on BiPASS: Aberrant activation of BiPASS and beyond. The BAG3 P209L mutation partially impairs BAG3-HSPB8 binding and leads to the aberrant activation of BiPASS. We hypothesize that BAG3 P209L outcompetes BAG1 for Hsp70-ubiquitinated client-complexes that were targeted for proteasomal degradation and reroutes these to the autophagy-lysosome system. Although BAG3 P209L seems to retain the ability to up-regulate the autophagic flux, client processing seems to be impaired, which leads to the accumulation of Hsp70 and associated proteins into BAG3 P209L-positive, ubiquitinated, punctae.

3.4 Potential Targets for Future Research

Future experiments should include testing the hypotheses assessed in Chapter 5 in cultured cardiomyocytes, mouse models, and patient-derived fibroblasts. In addition, several other questions remain yet to be assessed to clarify the exact functions BAG3 fulfils in autophagy and muscle cell maintenance and the exact cellular effects of the BAG3 P209L-mutation.

3.4.1 HDAC6; Another player in BiPASS?

Another interesting candidate that could be potentially involved in BiPASS and/or the BAG3-functions in myocyte maintenance is HDAC6. Research indicates that HDAC6 de-acetylates Hsp90 and competitively binds ubiquitinated, proteasomal Hsp90-clients and dynein,^{22,40–42} thereby promoting the autophagic degradation of multiple proteins in a chaperone-dependent manner in a process reminiscent of BiPASS. Furthermore, HDAC6 is required for the fusion of autophagosomes to lysosomes because it is responsible for the cortactin-dependent recruitment of the F-actin network.^{22,43} The literature on whether or not BAG3 and HDAC6 (temporarily) function in the same autophagy-lysosome pathways is divided. Whereas they are displayed as parts of the same complex in some instances,¹⁷ they are referred to as distinct processes in others.⁴⁴ Unlike BAG3, HDAC6 seems to function down-stream of autophagy induction and is not required for starvation-induced autophagy (BAG3 functions in ubiquitin-dependent and ubiquitin-independent pathways).^{2,22} However, it would be interesting to investigate whether HDAC6 is involved in the autophagosomal-lysosomal fusion in BiPASS, and if it is involved, at which point of the process it joins the complex.

Pull-down assays and fluorescent imaging studies in mammalian cells as done in Chapter 5 could help elucidate questions such as: Is HDAC6 present in BAG3-complexes (pull-down assays)? Is the association of HDAC6 with BAG3-complexes changed by the P209L-mutation? Does BAG3 P209L affect cellular HDAC6-localization? Does HDAC6 function adequately or is the cortactin-dependent recruitment of F-actin networks disrupted in cells expressing BAG3 P209L (to be assessed in cardiomyocytes or BAG3 P209L-patient derived fibroblasts)? Does the down-regulation of HDAC6 or the expression of a defective HDAC6-mutant exacerbate the BAG3 P209L phenotype? Does over-expression of HDAC6 rescue the BAG3 P209L punctae phenotype?

3.4.2 BAG3; Involved in F-actin Network organization?

Interestingly, BAG3 is also known to be required for the association of Hsp70 and the F-actin capping protein CapZ β 1 in muscle cells.⁴⁵ CapZ β 1 regulates the assembly of myofibrils and localizes to the Z-disc of muscle cells, thereby facilitating myofibril growth (and repair) under conditions of mechanical stress. Mechanical stress leads to the up-regulation of BAG3 as well as CapZ β 1 and their association Hsp70-positive complexes in muscle cells. Without BAG3, CapZ β 1 is degraded by the proteasome and cannot properly associate with Hsp70 and is not properly linked to the F-actin network, thus fibril remodeling is insufficient, resulting in fibril Z-disc instability and myofibrillar disintegration.⁴⁵ What is not known is whether these functions in the organization of the F-actin network are also important for the fusion of autophagosomes with lysosomes. It would be interesting to investigate the status of these F-actin networks in myocytes derived from patients with the BAG3 P209L-mutation.

To summarize, in muscle cells, CapZ β 1 is a proteasomal client that is directed to the Z-disc under conditions of cellular stress via BAG3 and associates with Hsp70. This complex then facilitates the association of CapZ β 1 with F-actin, thus mediating the formation and repair of myofibrils. Given the functions of HDAC6 discussed above and its functions in facilitating the recruitment of the F-actin network, HDAC6 would certainly be in a good position to facilitate or act in sequence with the association of CapZ β 1 and F-actin. Although it might be a bit of a long shot, it would be interesting to investigate whether expression of HDAC6 in BAG3 P209L-derived myocytes is capable of (partially) rescuing the devastating disease phenotype and re-instate the stability of the Z-disc. Most likely, BAG3 P209L punctae are eventually physically too large and capture too many tertiary proteins and factors for any rescue to persist because these effects are mediated through Hsp70 (see the effects of the dBAG mutant in Chapter 5). However, given the parallels between BAG3 and HDAC6 in their cytoskeletal functions, it would be interesting to investigate whether HDAC6 is capable of partially compensating for these defects and reinstate Z-disc integrity and/or F-actin mediated autophagosome-lysosome fusion.

4. Chaperone specificity;

A specific chaperone BAR-code for every toxic protein species?

It has been well documented that acute stress leads to the unfolding of normally folded proteins and the activation of the heat shock response (HSR), which leads to the up-regulation of molecular chaperones and a general increase in protein folding and protein degradation. Less is known about the PQC in chronic stress conditions, such as neurodegenerative diseases (NDDs), which tend to be caused by the accumulation of toxic protein species. Below, I will address if and how the Hsp70-system reacts to neurodegenerative diseases in post mortem brain tissue (see 4.1) and how well the various HSP-family members handle different disease-associated proteins (see 4.2).

4.1 Does the Hsp70-system recognize ataxin 3 aggregates in patients with Spinocerebellar ataxia type 3 (SCA3)?

To investigate whether members of the Hsp70-machine locate to polyglutamine aggregates in humans suffering from neurodegenerative diseases, we investigated the post-mortem brain tissue of patients with SCA3 in Chapter 3.⁴⁶ Using immunohistochemistry and microscopy, we quantitatively analyzed the different forms of protein aggregates in the pons of SCA3 patients in relation to differential expression levels of the components of the Hsp70-machine. Our findings indicate that a sequence of events takes place during the aggregation process in SCA3 and that the components of the Hsp70-machine only respond to and become impaired at a late stage in this process.

4.1.1 *The evolution of aggregate formation in SCA3*

Our experiments in Chapter 2 (summarized in Table 1), indicate that polyglutamine elongated ataxin 3 accumulates according to a distinct sequence of events: Granular cytoplasmic aggregates, visible as granular cytoplasmic polyQ staining (GCS), move into the nucleus, where they are visible as diffuse nuclear staining (DNS). Following their entry into the nucleus, ataxin 3 accumulates into on or at most two distinct neuronal nuclear aggregates (NNI), which we hypothesize to be the final stage of aggregation that ultimately results in neuronal cell death.⁴⁶ A similar sequence of events was recently proposed on the basis of analysis of brains from Huntington patients.⁴⁷

| | Aggregate staining pattern | p62-staining | UBB ⁺ -staining | HSPA1A up-regulated |
|---------|----------------------------|----------------|----------------------------|---------------------|
| Stage 1 | GCS | Rarely | None | No |
| Stage 2 | DNS | Rarely | None | No |
| Stage 3 | NNI | Constitutively | None | No |
| Stage 4 | NNI | Constitutively | Some | No |
| Stage 5 | NNI | Constitutively | Constitutively | Yes |

Table 1: This table displays a sequence of events in SCA3, which we propose proceeds in stages.

Table 1 also shows, that the cellular aggregation markers p62 and ubiquitin do not react to ataxin 3 aggregation until late in the process: On the one hand, although p62 seemed to start reacting to granular cytoplasmic and diffuse nuclear ataxin 3 aggregates, it seemed to have clear preference for NNIs. On the other hand, the marker for proteasomal overload, Ubb⁺, was only present in a subset of NNI containing neurons, which suggests that a fraction of neurons with NNIs may exhibit signs of proteasomal dysfunction and that GCS and DNS most likely represent earlier, less stressful stages of the aggregate pathology.

4.1.2 The Hsp70-system in SCA3

Next to aggregation markers, we monitored the expression of the stress-inducible chaperones HSPA1A and DNAJB1 in post-mortem brain tissue of SCA3 patients.⁴⁶ To our surprise, we found that HSPA1A was only up-regulated in a subset of neurons containing p62- and UBB⁺-positive NNIs. DNAJB1 seemed to be trapped in NNIs in these neurons, which generally showed morphological abnormalities indicative of poor cellular health. These findings indicate that, although the HSR is still active in neurons expressing mutant ataxin 3, it is apparently not properly activated in the early stages of neuronal ataxin 3-aggregation. This is likely because mutant ataxin 3 is not recognized as misfolded protein per se, instead HSPA1A up-regulation has most likely no cytoprotective effects but demarks the final stage of the progressive SCA3 aggregation pathology.

4.2 Are all chaperones equally effective in handling cellular toxicity conveyed by disease-associated aggregation-prone proteins?

Our findings that the HSR is either not or insufficiently activated in neurons affected by protein aggregation in SCA3 patients led us to perform a literature research on the effectiveness of various chaperones in handling neurodegeneration-associated protein aggregates (Chapter 3). By grading the published data on chaperone efficiency against proteinopathy-associated aggregates in different model organisms, we found a different BAR-code of chaperones were effective against different proteinopathy-associated proteins. Overall, our results indicate that, the proteins up-regulated by the HSR tend to be somewhat effective in preventing protein aggregation in in vitro and in mammalian cells. However, whereas they seem to be effective in organismal model organisms for amyloid-beta-, tau-, and androgen receptor-induced aggregation, they are mostly insufficient in buffering the toxic effects conveyed of polyQ-aggregates. Instead, certain DNAJs (e.g. DNAJB2, DNAJB6) can effectively ameliorate aggregate-toxicity in longer lived animal organisms for diseases such as HD and SCA3 ⁴⁸⁻⁵¹

Taken together these data raise the intriguing hypothesis that canonical chaperones, such as the Hsp70- and Hsp90-refolding cycles might be well equipped to compensate for certain cellular homeostatic imbalances but unable to handle polyQ-aggregates themselves, whereas on-canonical chaperones, such as certain DNAJs, HSPBs, and maybe NEFs might be better equipped to handle polyQ-aggregates by shielding and ultimately re-directing the toxic protein –or any of it's pre-aggregate forms- to cellular degradation systems.

5. Cell autonomous versus non-cell autonomous effects in neurodegenerative diseases: A role for chaperones?

5.1. Neuronal vulnerability:

Chaperones and cell autonomous protection

Interestingly, not all NDDs are affecting the same subtypes of neurons and thus result in a different set of symptoms (reviewed in Chapter 3). The specific

localization and characteristics of neuronal cell types determine their vulnerability to specific protein aggregation diseases.^{52,53} These characteristics may include differential expression levels of the disease-associated protein in diverse neuronal subtypes and the exposure and reaction to various stresses and exogenous influences (e.g. neurotransmitters in neurons) that may act as triggers for the aggregation of the respective disease-associated proteins. For instance, on the one hand, alpha-synuclein aggregates have been associated with neuronal degeneration in the basal ganglia, which results in dopamine deficiency and motor symptoms, including tremors, muscular rigidity, and slow and imprecise movements. On the other hand, accumulation of amyloid beta and tau result in neurodegeneration of the dental gyrus and hippocampus, causing progressive mental degradation, including memory deficits.^{49,52,53}

Our laboratory is particularly interested in Huntington's disease and SCA3, both of which are polyQ-driven diseases. Although the causative proteins are ubiquitously expressed across all cell types, hypersensitivity to aggregation and toxicity seems to primarily involve the neurons of the basal ganglia for HD⁵⁴ and the Purkinje cells in the Cerebellum for SCA3⁵⁵. This implies that triggers specific to these brain areas may be required to initiate aggregation of disease-associated proteins. What these specifically are and whether differential expression of HSPs may protect against these trigger factors is yet to be investigated.

5.2. Involvement of non-neuronal cells:

Chaperones and cell non-autonomous effects

In HD as well as in SCA3 the disease seems to spread from one specific brain area (the basal ganglia in HD, the cerebellum in SCA3) to the rest of the brain in a pre-meditated pattern. Interestingly, similar specific progressive spreading patterns have been observed in most neurodegenerative diseases⁵⁶.

As discussed in Chapter 1, it has been suggested that toxic protein species can convert other, structurally related non-toxic proteins, into toxic protein species (aggregates), thereby seeding aggregation in adjacent neurons.^{56,57} This phenomenon (details in Chapter 1) is thought to lead to the propagation of toxic protein species from one neuron to the next, thereby leading to progressive neurodegeneration and the associated symptoms. In how far polyQ diseases are also prion-like diseases is still a matter of debate.^{56,58}

Another (related) debate in polyQ diseases concerns the role of non-neuronal cells in the progression of the disease. While neurons are generally considered to be the most vulnerable brain cells, recent data suggest that glial cells may be a contributor to neurodegenerative polyQ-diseases as well.^{59–61} Literature indicates that astrocytes are generally less affected by polyQ-toxicity and contribute to disease via more indirect mechanisms.⁶⁰ Interestingly, immunohistochemical analyses of post-mortem brain tissue from patients with various neurodegenerative diseases indicate that several HSPs, like DNAJB6 and HSPBs, tend to be up-regulated in the astrocytes of disease-affected brain areas.^{62,63} We took this up-regulation of HSPs in astrocytes as incentive to hypothesize that astrocytes might be able to protect neurons from polyglutamine toxicity in a non-cell autonomous manner; by supporting neuronal fitness (supplying chemokines, nutrients, or even transmitting chaperones) or by interfering with neuron-to-neuron transmission of prion-like polyglutamine aggregates.

To investigate whether the astrocytic expression of DNAJB6 can ameliorate neuronal polyQ-initiated degradation, we generated transgenic *D. melanogaster* lines that independently expressed disease-causing Htt in neurons and DNAJB6 in astrocyte-like cells (Chapter 4). Our experiments showed that DNAJB6 could alleviate mHtt-mediated neurodegeneration cell-autonomously and non-cell autonomously: Neuronal and astrocytic expression of DNAJB6 prolonged lifespan in an HD *D. melanogaster* model.

Our data did not support the idea that DNAJB6 is transferred from astrocyte-like cells to neurons,⁶⁴ instead, our data support the notion that astrocytic DNAJB6-expression protects neurons from polyQ-induced toxicity in a cell non-autonomous manner: By taking up extra-cellular aggregates. Such data elaborate on recent findings from the Kopito group, who showed that aggregates from chemically-destroyed neurons is to be found up in neighbouring astrocytes.⁵⁸ How DNAJB6 expression in astrocytes enhances this phenomenon still remains unclear. One possibility is that DNAJB6 prolongs the astrocytic ability to provide distressed neurons with nutrients and neuroprotective factors. Alternatively or in parallel, DNAJB6 may enhance the astrocytic ability to take up toxic extra-cellular polyQ species. Cellular experiments from our laboratory indicate that immortalized astrocyte-like cells can take up more extracellular polyQ-peptides than immortalized neuron-like cells, indicating that astrocytes may function as encephalic vacuum cleaners.^{Data not published} Further cellular experiments to elucidate the mechanism underlying DNAJB6-mediated non-cell-autonomous protective

effects could include kinetic measurements on polyQ-peptide uptake and on seeding potential and toxicity of internalized aggregates.

6. Conclusion

To conclude, the experiments presented in this thesis aimed to shed more light on the intricate organization of the protein quality control network by studying protein aggregation in neurodegenerative diseases and myofibrillar myopathies. The most pertinent conclusions are:

- The HSR is not activated by mutant polyglutamine proteins, which means that the observed decline in HSR is a consequence rather than a cause of disease (see Chapter 2).⁴⁶
- Not all aggregates are the same; Handling of each aggregate requires a different set of HSPs (see Chapter 3).⁴⁹.
- PolyQ-aggregates can propagate from neurons to astrocytes in *D. melanogaster*; The expression of human DNAJB6 has protective cell autonomous as well as protective non-cell autonomous effects against polyQ-aggregates. This DNAJB6-mediated increase in astrocytic resistance to polyQ-seeds likely also positively affects neuron-to-neuron progression of polyQ-aggregates, thereby protecting neurons in adjacent areas from polyQ-infection and thus delaying the progression of neurodegeneration.
- Chaperones are not only key to rescue protein aggregation disease but subtle defects in PQC, as in the case of BAG3 P209L can have devastating effects on cellular fitness and cause diseases, highlighting the importance of a proper PQC for a healthy life span.

To summarize what working on this thesis has taught me in one sentence:

Control your proteins, livelong, and prosper.

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